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Diagnosis of von Willebrand Disease Type 2N: A Simplified Method for Measurement of Factor VIII Binding to von Willebrand Factor

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Diagnosis of von Willebrand disease Type 2N (vWD 2N), which mimics hemophilia A and its carrier state, is important for accurate genetic counseling and appropriate therapy. To make testing for the disorder more clinically applicable, we developed a simplified method for measurement of factor VIII (FVIII) binding to von Willebrand factor (vWF) using commercially available reagents and standard clinical assays. FVIII binding to vWF was measured by capture of patient vWF by polyclonal antibodies on cyanogen bromide-activated Sepharose beads, reaction with recombinant FVIII, and assay of unbound FVIII by clotting methods. Unbound vWF was measured in patient plasma after capture by the Laurell method. The ratio of bound FVIII/bound vWF was normal in hemophilia A, vWD Type 1, and vWD Type 3 patients, and abnormal in 5 subjects from two families, all of whom had vWD 2N mutations. Patient 1, with FVIII 8 U/dl, vWF: Ag 61 U/dl, vWF:RC 74 U/dl, and FVIII binding nil, was homozygous for the Arg91Gln mutation. She was followed during pregnancy and delivered an unaffected heterozygous son. Patient 2 had FVIII 8 U/dl, vWF:Ag 73 U/dl, and vWF:RC 71 U/dl, and very low FVIII binding. She was heterozygous for Arg91Gln, as were her mother and sister; no second vWD 2N mutation was found. Her brother, with FVIII 14 U/dl, vWF:Ag 113 U/dl, and vWF:RC 72 U/dl, has no evidence of vWD 2N. With an X-linked inheritance pattern of bleeding tendency, this family is the first reported with combined hemophilia A and vWD 2N. Am. J. Hematol. 58:311–318, 1998. © 1998 Wiley-Liss, Inc.

Key words: von Willebrand disease; vWF mutations; factor VIII

INTRODUCTION

Von Willebrand disease (vWD) is a common disorder of hemostasis caused by qualitative or quantitative defects in von Willebrand factor (vWF). vWF, an adhesive glycoprotein, serves two functions in the hemostatic process. In formation of the platelet plug, vWF is required for platelet adhesion to subendothelium and platelet aggregation. vWF also acts as a carrier for factor VIII (FVIII), stabilizing it and localizing it to the site of vascular injury [1]. FVIII is often reduced in vWD secondary to decreased or defective vWF, in contrast to its primary reduction in hemophilia A. Clinically, distinction between vWD and hemophilia or its carrier state is based on measurements of vWF antigen and activity, platelet function, and bleeding time, all of which are within the normal range in hemophilia A.

Von Willebrand disease Type 2N (vWD 2N) is unique among the many subtypes of vWD [2] in that it often mimics hemophilia A or its carrier state [3–6]. In vWD 2N, FVIII is reduced due to decreased binding of FVIII by vWF [7–9]. In most cases, mutations have been found in exons 18–20 of the vWF gene [10–12], which code for the FVIII binding site [13,14], although mutations in other regions have recently been described [15]. Inheritance of two vWF mutations appears to be required to produce the vWD 2N phenotype. The disorder has been reported in patients who are homozygotes or compound

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heterozygotes for FVIII binding mutations [3,6–8,15–20] and in patients heterozygous for a vWD 2N mutation who have a second demonstrated or presumed mutation elsewhere in the vWF gene [6,9,15,17,21–26]. Patients in the former category often have low FVIII but normal levels of von Willebrand factor antigen and activity, bleeding time, and ristocetin induced platelet aggregation [3,6–8,16,17,19]. The lack of symptoms in their parents and offspring suggests autosomal recessive inheritance. Distinguishing such vWD 2N patients from individuals with hemophilia genes is important for purposes of genetic counseling and for therapy, since treatment products for vWD 2N should contain normal vWF [5].

Methods described for measurement of FVIII binding to vWF, based primarily on that of Nishino and colleagues [7], are complex and costly, requiring use of monoclonal anti-vWF on microtiter plates, chromogenic assay of bound FVIII, and ELISA for bound vWF. The Scientific and Standardization Committee of the International Society on Thrombosis and Haemostasis in an international survey found only 16 laboratories worldwide performing testing for vWD 2N by measurement of FVIII binding to vWF and described the method as cumbersome [12]. They recommended that testing be performed in all patients with a FVIII deficiency not clearly linked to the X chromosome. We describe a simplified method for measurement of FVIII binding to vWF using commercially available reagents and providing quantitative results and report diagnosis of vWD 2N in two families ascertained through ''hemophilic'' females, one of whom was followed during pregnancy. One family appears to be transmitting both vWD 2N and mild hemophilia A.

MATERIALS AND METHODS

Subjects

Subjects studied included normal individuals and patients with a diagnosis of hemophilia A, Type 1 vWD, and Type 3 vWD. Two subjects considered to be female hemophiliacs or ''symptomatic carriers'' of hemophilia A who were found to have vWF mutations are described below.

Patient 1 (MG) is a 25-year-old women with a lifelong history of bleeding. She was diagnosed as having FVIII deficiency at age 12 prior to surgery for scoliosis. Harrington rods were placed under FVIII concentrate coverage. Other surgeries included removal of a breast lump, nasal surgery, and laparoscopy. She has had epistaxis, bruising, and minor bleeding episodes, which have been controlled with intravenous or intranasal desmopressin. Menorrhagia has been controlled with the use of oral contraceptives, which she discontinued to become pregnant. The patient was adopted as an infant through a private adoption. In order to clarify the risk to her fetus, her birth mother was contacted. The birth mother reported no family history of hemophilia. She had experienced no bleeding from surgeries but heavy bleeding post deliveries. Among her five other children, a son and a daughter had excessive bleeding with tonsillectomy but have not been studied. The father's history is not known. The patient was negative for the FVIII inversions commonly seen in severe hemophilia A.

Patient 2 (SO) is the sister of a 10-year-old boy diagnosed as having mild hemophilia A. She presented at age 18 for genetic counseling regarding hemophilia. Records supplied by the family revealed that a carrier test performed on her through her local physician 2 years previously showed a factor VIII level of 7% and a vWF antigen of 67%. Her parents had been informed only that she was a hemophilia carrier. The patient had experienced chronic joint problems related to sports activities and had been forced to withdraw from some sports because of injuries. Subsequent to her first testing she experienced a large frontal hematoma with periorbital ecchymosis after bumping heads with another person. Her bleeding episodes went untreated. A trial of intravenous desmopressin showed a sixfold rise in factor VIII. Her sister had FVIII level of 22% and vWF:Ag of 126%. Her history was negative except for epistaxis. Her brother is a 10-year-old boy diagnosed as having mild hemophilia A at age 7 following knee trauma. He had undergone circumcision with no complications; no other surgical procedures had been performed. His trial of intravenous desmopressin showed a threefold increase in factor VIII and twofold increases of vWF antigen and activity. The parents have no history of excessive bleeding.

Methods

Sample preparation. Blood was collected in 3.8% sodium citrate. Platelet-poor plasma was separated by centrifugation at 2,000*g* for 15 min at 4°C and stored in aliquots at −70°C. The cell pellet was frozen at −70°C for DNA extraction.

Factor VIII and vWF assays. Factor VIII activity (FVIII) was measured by one-stage assay using immunoadsorbed FVIII-deficient plasma on an automated coagulation analyzer (STA, Diagnostica Stago, Asnieres, France). Von Willebrand factor antigen (vWF:Ag) was measured by Laurell quantitative immunoelectrophoresis in agarose gels using polyclonal antisera (Diagnostica Stago, Asnieres, France). Von Willebrand factor activity was measured as ristocetin cofactor (vWF:RC) by agglutination of formalin-fixed platelets (BioPool International, Ventura, CA) by ristocetin in an impedance aggregometer (Chronolog, Havertown, PA). Reference plasmas used were the College of American Pathologists' (CAP) Fibrinogen, Factor VII, and Factor VIII Related Substances Reference Material (College of American Pathologists, Northfield, IL) and Universal Coagu-

TABLE I. Primer Sequences for vWF Mutations Affecting Factor VIII Binding

Mutation	Enzyme	Primers		Fragment size
Arg91Gln	MspI	5′TGT GTT CCT TCA TTG CCT CC3′	Mutation:	265 + 118
		5′AAT GTG GAC TAG CAG TAG CC3′	Normal:	167 + 118 + 98
Arg53Trp	HpaII	5′AGT TGA CAG GGA GGA GCC AT3′	Mutation:	474 or 376 + 98
		5′AGG CAG AGA GTA ACC AGG TT3′	Normal:	251 + 223 or 251 + 125 + 98
Thr28Met	NlaIII	5′CCT GCC TAC AAG AAA ACT GAA3′	Mutation:	54 + 36 + 27 + 22 + 6
		5′AGG GCT CGA GTG TAC CAA CA3′	Normal:	54 + 49 + 36 + 6
His54Gln	NlaIII	5′GGG TTT AGA TCA GTC ACT GTG3′	Mutation:	155 + 63
		5′GTG CAC CCT CAC TCC ACC CGC3′	Normal:	218
Arg19Trp	BspMI	5′ACT ACT TCT TTG CTT TTC CTA3′	Mutation:	244
		5′CCT GCC TAC AAG AAA ACT GAA3′	Normal:	148 + 96
Glu24Lys	TaqI	5′ACT ACT TCT TTG CTT TTC CTA3′	Mutation:	219 + 25
		5′CCT GCC TAC AAG AAA ACT GAA3′	Normal:	139 + 80 + 25

lation Reference Plasma (UCRP) (Pacific Hemostasis, Huntersville, NC).

FVIII binding assay.

Preparation of anti-vWF Sepharose. CNBr-activated Sepharose 4B (Amersham Pharmacia Biotech, Piscataway, NJ) was suspended in cold 1 mM HCl in a test tube and gently rotated for immediate swelling. For 15 microcentrifuge tubes, 1.2 g of Sepharose in 7 ml of HCl were used. The gel was then washed with 25 ml of 1 mM HCl, centrifuged at room temperature at 5,000 rpm for 15 min, and the supernatant slowly decanted and discarded. The washing procedure was repeated three times. Polyclonal rabbit anti-human vWF antiserum (Diagnostica Stago, Asnieres, France) was diluted in coupling buffer (250 mM $NaHCO_3$/ 500 mM NaCl, pH 9.0) at a concentration of 0.5–0.75 U/ml and incubated with the washed Sepharose for 1–2 hr on an orbital shaker. The mixture was centrifuged as above and the pellet was washed twice with five volumes of coupling buffer. The pellet was resuspended in 30 ml of 1M ethanolamine, pH 8.0, and incubated on an orbital shaker for 2 hr. After centrifugation, the anti-vWF Sepharose was washed with two alternating cycles of 100 mM sodium acetate/0.5M NaCl, pH 4.0, and 0.1M Tris-HCl/0.5M NaCl, pH 8.0, at 5X gel volumes. After washing, the pellet was resuspended in 7 ml Owren's veronal buffer (OVB) (Diagnostica Stago, Asnieres, France). Aliquots (450 μl) were added to 1.5-ml microcentrifuge tubes. Pellets were washed three times by filling tubes with OVB, spinning at 4,000 rpm for 5 min at 4°C, and discarding supernatants. Prepared tubes were stored at 4°C.

Capture of patient vWF. Four hundred microliters of standard or test plasma diluted in OVB were added to the tubes and incubated at 37°C for 2 hr with shaking. Tubes were then centrifuged at 4,000 rpm for 5 min at 4°C, and the supernatants were removed and assayed for vWF:Ag.

Removal of patient FVIII. Pellets were washed twice in OVB. Pellets were resuspended in 1 ml of 0.4 mM $CaCl_2$ and incubated for 30 min at 37°C with shaking. Tubes were then centrifuged and the pellets washed twice with OVB.

Reaction with FVIII. Recombinant FVIII (Kogenate, Bayer, West Haven, CT) was reconstituted with imidazole-buffered saline with 1% BSA, pH 7.4, at 1 U/ml and stored at −70° until use. After thawing, 1 ml was added to each pellet and incubated at 37°C for 5 min. Aliquots were removed and centrifuged at 6,000 rpm for 6 min at 4°C. Supernatants were removed to plastic tubes on ice. FVIII activity of supernatants was measured using imidazole-buffered saline for dilution.

Quantitation of FVIII binding. Bound FVIII for each dilution of standard or patient plasma was calculated as added FVIII (1.00 U/ml) minus remaining supernatant FVIII. A standard curve was prepared using reference plasma undiluted and diluted 1:2, 1:5, 1:10, and 1:20 in OVB. The least-squares linear regression equation for bound FVIII against logarithm of concentration was calculated for the standard and used to calculate concentration for each patient plasma dilution. Patient plasma was tested undiluted and diluted 1:2 in OVB. Bound vWF was calculated as plasma vWF:Ag minus vWF:Ag remaining in supernatant after capture of patient vWF. FVIII binding was expressed as the ratio of bound FVIII to bound vWF.

Mutation and polymorphism analysis. DNA was isolated from peripheral blood leukocytes by standard methods. PCR amplification was performed on an aliquot of the purified DNA in an automated thermal cycler, using oligonucleotide primers (Macromolecular Resources, Fort Collins, CO) and GeneAmp PCR Core Reagents (Perkin Elmer Cetus, Norwalk, CT). Amplified DNA was incubated with restriction enzymes (New England Biolabs, Beverly, MA), as recommended by the supplier. Digested fragments were run on agarose gels containing ethidium bromide, visualized under UV, and interpreted by comparison with DNA size markers. vWD 2N mutations were detected using the primer sequences shown in Table I [25] (Tuley, personal communication). Polymorphisms in the vWF [27] and FVIII [28] genes were also analyzed by similar methods.

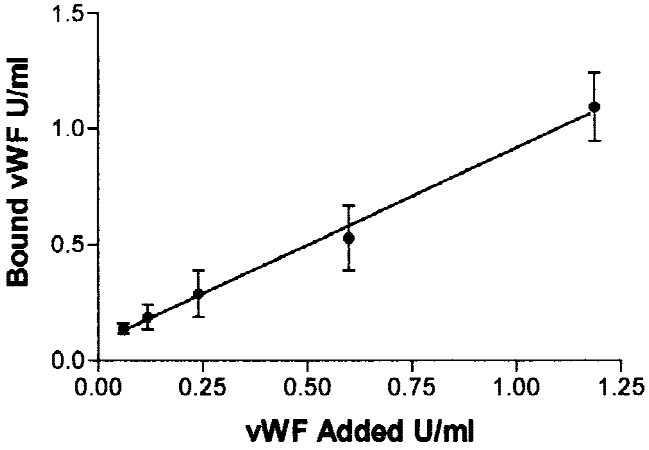

Fig. 1. Binding of plasma von Willebrand factor to immobilized rabbit anti-human vWF. Means of 5 assays using two different reference plasmas (vWF:Ag = 1.16–1.19 U/ml) are shown with error bars representing standard deviations.

RESULTS

Plasma vWF was shown to bind to antibody-bearing Sepharose in amounts proportional to the amount of vWF added, as determined by measurement of residual vWF:Ag in supernatant plasma (Fig. 1). When plasma FVIII was dissociated from vWF with $CaCl_2$, and recombinant FVIII at a single concentration (1 U/ml) was added, FVIII removed from the supernatant and presumably bound to immobilized vWF was proportional to the amount of vWF in reference plasma added (Fig. 2A). In control mixtures without vWF or without antibody, nonspecific binding of FVIII to the antibody or to Sepharose was negligible. The binding followed a hyperbolic one-site binding curve. Transformation of dilution to logarithms produced a curve that was linear between undiluted and 1:20 for pooled normal plasma, two different reference plasmas (CAP reference, vWF:Ag = 1.16 IU/ml, and Universal Coagulation Reference Plasma, vWF:Ag = 1.19 IU/ml), and normal individuals. An example of the standard curve is shown in Figure 2B.

Results of tests performed on normal subjects and patients with diagnoses of mild or moderate hemophilia A, vWD Type 1, and vWD Type 3 are shown in Figure 3 and Table II. The ratio of bound FVIII to bound vWF in normal subjects had a mean of 0.99 and a range of 0.70 to 1.29. Two standard deviations about the mean gave a normal range of 0.65–1.32. The bound FVIII/bound vWF ratio was below the normal range in five subjects from two families, all of whom were found to have vWD 2N mutations. Data on these patients and their families are shown in Table III.

Patient 1 was found to be homozygous for a previously described mutation in exon 20 of the vWF gene, Arg91Gln, which is the most common mutation seen in

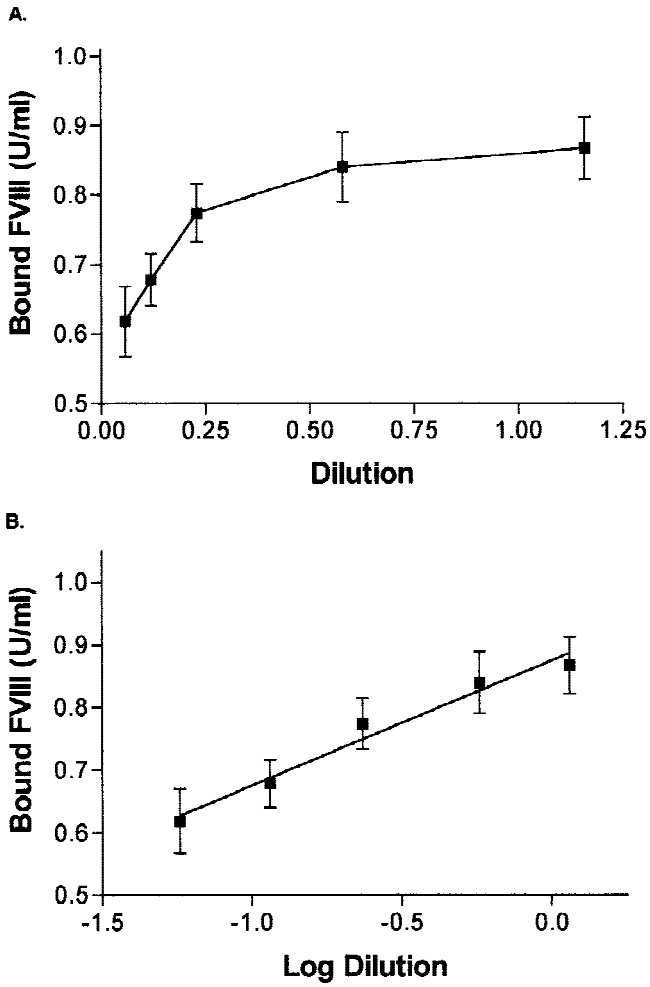

Fig. 2. A: Binding of recombinant factor VIII to immobilized plasma von Willebrand factor. Means of 7 assays using two different reference plasmas are shown with error bars representing standard deviations. B: Example of standard curve generated by factor VIII binding assay. Linear regression of bound FVIII against logarithm of dilution of reference plasma.

vWD 2N [12]. When she presented in early pregnancy, her FVIII and vWF levels were not significantly different from her previous studies (Table III). By the third trimester, her FVIII had risen to 34 U/dl with vWF:Ag of 246 U/dl and ristocetin cofactor of 150 U/dl; her FVIII binding remained undetectable (Fig. 4). The patient delivered without complications under treatment with Humate P (Centeon, King of Prussia, PA). Her son, heterozygous for Arg91Gln, has FVIII binding intermediate between that of his mother and the normal subjects and more than 2 standard deviations below the mean of the normal subjects. Plasma from the patient's birth mother was not available for binding studies. Her results reported from another laboratory showed a reduced ratio of FVIII activity to vWF:Ag.

Patient 2, her sister, and her mother were heterozygous

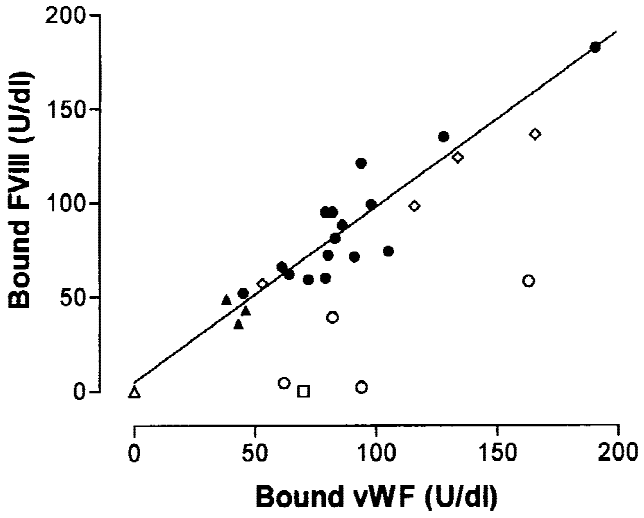

Fig. 3. Factor VIII binding assay results in normal subjects (●), hemophilia A subjects (♢), vWD Type 1 subjects (▲), vWD Type 3 subjects (△), vWD Type 2N homozygote: Patient 1 (□), and vWD Type 2N heterozygotes: son of Patient 1, Patient 2, sister of Patient 2, and mother of Patient 2 (○).

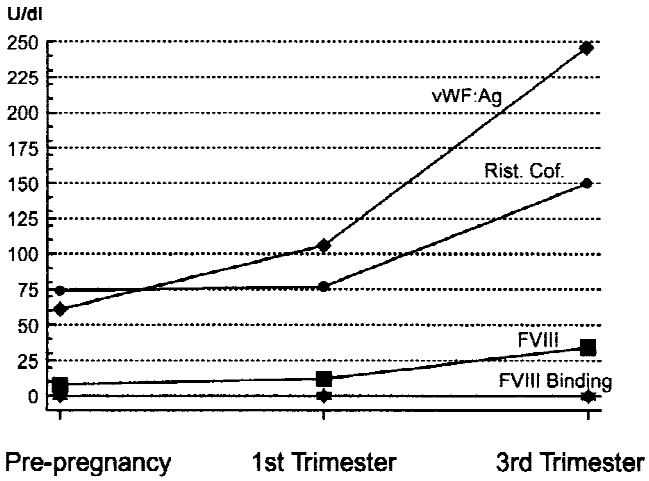

Fig. 4. Changes during pregnancy of Patient 1 in factor VIII (FVIII), von Willebrand factor antigen (vWF:Ag), ristocetin cofactor (Rist. Cof.), and FVIII binding.

TABLE II. Factor VIII Binding Assay Results in Normal Subjects, Patients, and Family Members

Subjects	BOUND FVIII (U/dl)	BOUND vWF (U/dl)	Ratio FVIII/vWF
Normals	52–142	45–188	.70–1.29
Hemophilia A	57–136	53–166	.82–1.08
vWD Type 1	36–49	38–43	.84–1.29
vWD Type 3	0–3	0	—
Patient 1 (MG)	0	70	0
Son	59	163	.36
Patient 2 (SO)	2	62	.03
Brother	98	116	.84
Sister	1	94	.01
Mother	39	82	.48
Father	142	188	.76

for Arg91Gln (Table III and Fig. 5A). Her brother, despite a low level of FVIII, was negative for vWD 2N mutations and showed normal FVIII binding, indicating that he has mild hemophilia A. Haplotypes of the vWF gene (Fig. 5B) show that the two sisters received the same vWF allele from their mother, designated ''m,'' which presumably bears the vWD 2N mutation, while their brother received her other vWF allele. All three siblings received the same paternal vWF allele, which in both father and son shows no evidence of producing a binding defect. The X-linked inheritance pattern of bleeding in the mother's family supports the presence of a FVIII mutation. Inheritance of the *Bcl*I polymorphism in the FVIII gene indicates that both sisters received the same FVIII allele as their affected brother and are thus likely to be hemophilia carriers in addition to their vWF defect, as in their mother. The sisters, however, have much lower FVIII binding than the mother.

TABLE III. Laboratory Findings in Families With vWD 2N Mutations

Subject	FVIII (U/dl)	vWF:Ag (U/dl)	vWF:RC (U/dl)	FVIII binding ratio	vWF mutations identified
Patient 1					
Pre pregnancy	8	61	74		Arg91Gln Homozygote
1st trimester	12	106	77	0	
3rd trimester	34	246	150	0	
Son					
Cord blood	140	116	Not done	.36	Arg91Gln Heterozygote
Venipuncture	90	73	54		
Mother	56	94	108	Not done	Not done
Patient 2	8	73	71	.03	Arg91Gln Heterozygote
Sister	25	79	82	.01	Arg91Gln Heterozygote
Brother	14	113	72	.84	None
Mother	49	94	90	.48	Arg91Gln Heterozygote
Father	138	183	94	.76	None
Normal range	50–200	45–200	45–200	.65–1.32	

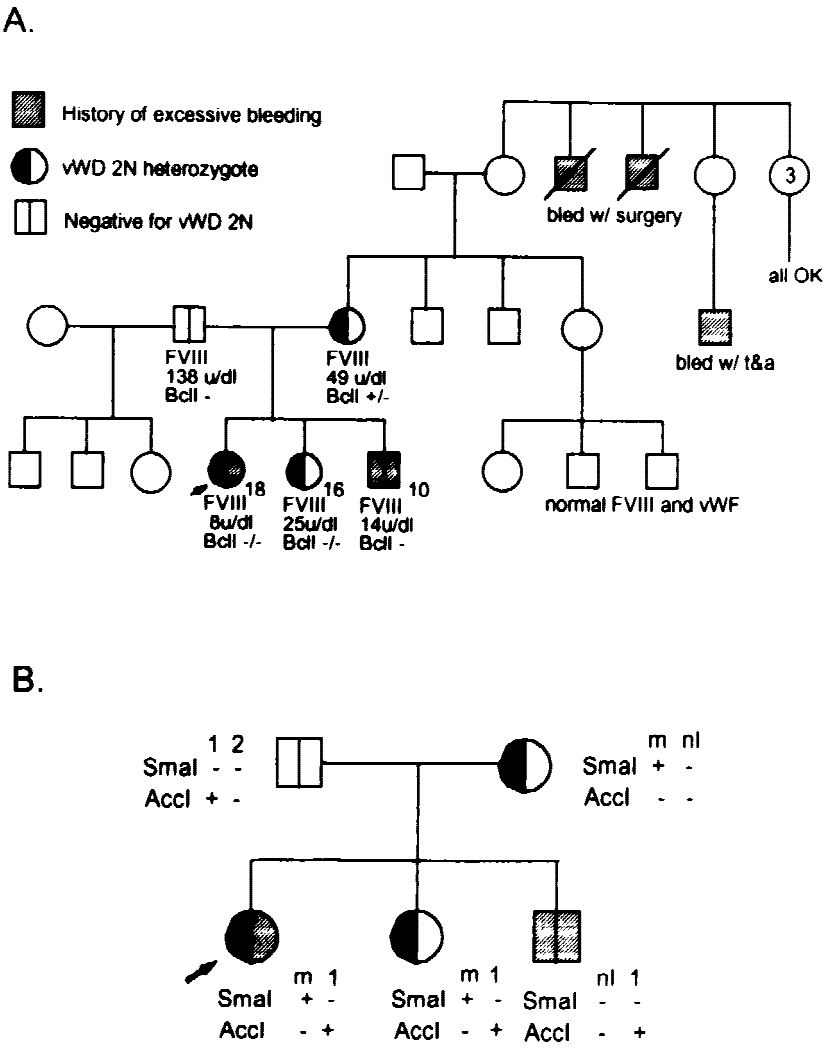

Fig. 5. Family of Patient 2. A: Pedigree with bleeding history, FVIII levels, and FVIII gene polymorphism. B: VWF gene haplotypes.

DISCUSSION

The method described by Nishino et al. [7] for demonstration of defects in FVIII binding to vWF has been important in diagnosis and characterization of vWD 2N. However, its complexity makes it difficult to apply under routine clinical conditions. We sought a simpler method with which to identify the disorder. Previous investigations [7,8] had shown that both homozygotes and heterozygotes could be clearly distinguished from normal individuals at multiple points along the binding curve, suggesting that a limited number of dilutions could be used if optimum conditions were identified. Vlot et al. [29] in kinetic studies of FVIII-vWF interaction used monoclonal antibody bound to cyanogen bromide-activated Sepharose and measurement of residual vWF and FVIII in the supernatants in quantitation of amount bound. They found that vWF from both normal and hemophilic plasma bound similarly to antibody-bearing Sepharose, as did vWF subfractions containing only high or low molecular weight multimers. Recombinant FVIII was found to bind to immobilized vWF in a concentration dependent manner. Our data support those findings.

The two groups who originally described patients with FVIII binding defects used purified plasma FVIII in their assays [3,7,8,17], but subsequent studies elsewhere have used recombinant FVIII [6,18,20,21,24,25], which is more readily available. Reported methods also used monoclonal rather than polyclonal anti-vWF antibodies and chromogenic assay for FVIII. The method we have described, while labor-intensive, minimizes use of costly reagents and methods not widely practiced in clinical laboratories in the United States. The test detects both homozygotes and heterozygotes for vWD 2N and provides quantitative results, facilitating clinical studies.

Presentation of a hemophilic female for genetic counseling, particularly one with no information on family history, presents a challenge. The possible causes for manifestation of an X-linked recessive disorder in a female have been reviewed [30]. They include chromosome aberrations, homozygosity, ‘‘extreme Lyoniza-

tion'' in a hemophilia carrier, and misdiagnosis of another disorder. vWD 2N is now of primary importance in the differential diagnosis. Distinguishing between X chromosomal and autosomal recessive inheritance is crucial for accurate genetic counseling. The hemophilia carrier may transmit hemophilia to her offspring regardless of the genotype of her partner. In vWD 2N, an autosomal recessive disease, mutations from both parents are required to produce the disease phenotype [12].

The phenotype of Patient 1 can be completely explained by her homozygosity for a vWD 2N mutation. However, hemophilia carrier status as well cannot be completely ruled out. She is negative for the FVIII inversions common in severe hemophilia A [31]. Patient 2 has a phenotype very similar to that of Patient 1, including a strikingly low level of FVIII binding, but to date no second vWF mutation has been identified in her. The presence of a normal amount of vWF with normal function suggests that both vWF alleles are expressed. She apparently also carries a mutation in the FVIII gene, which produced hemophilia in her brother; he has a similar level of FVIII but no FVIII binding defect. Her phenotype may, therefore, be due to a combination of a vWF and a FVIII defect. The mother and two daughters in this family, who are all double heterozygotes for vWD 2N and hemophilia A, have FVIII levels of 8, 25, and 49 U/dl. These phenotypic differences may reflect X-chromosome effects as seen in other hemophilia carriers.

This is the first report of a family with combined hemophilia A and vWD 2N, although the combination of hemophilia with vWD Type 1 has been observed [32]. vWD 2N mutations occur at a relatively high frequency. In The Netherlands [22], a heterozygote frequency of 1 in 50 was found, predicting that the disease in homozygous or compound heterozygous state should occur at a frequency of 1 in 10,000, making it more common than other Type 2 variants of vWD and 100 times more common than vWD Type 3 [33]. While the frequency of vWD 2N may not be as high in other populations, chance combinations of vWD 2N and hemophilia A are likely to occur and may result in diagnostic dilemmas. The presence of relatively common FVIII binding mutations contributes to the variability seen in vWD and may account for the clinical features of some ''hemophilic'' females.

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